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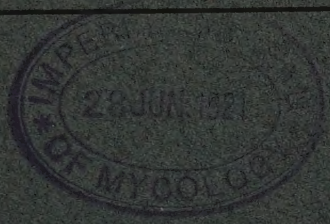

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DIAGNOSING WHITE-PINE BLISTER-RUST FROM ITS MYCELIUM

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INTRODUCTION

The mycelium of *Cronartium ribicola* Fischer, the causal organism of the white-pine blister-rust, can be found among the cells of *Pinus strobus* L. for some time before the spores of the fungus are produced. The examination of thousands of sections of diseased bark has convinced the writer that the morphological characters of the mycelium and its method of parasitism are sufficiently distinctive to warrant the assertion that the blister-rust can be recognized from the mycelium alone.

The stains generally employed in the preparation of slides for diagnosis are safranin and lichtgruen. The use of these two stains is, of course, already well established in both zoological and botanical work, and their employment in combination is by no means new. They are particularly valuable in the study of the blister-rust mycelium because they not only stain host and parasite at the same time but they also bring out extremely well the anatomical features of the host tissue. It has always seemed to the writer that more detailed accounts of the experiences of investigators in handling stains would be of very great value to students. Accordingly the method which has been employed is given below, step by step, with the hope that it may be useful and suggestive to investigators of both normal and pathological anatomy. Throughout, the writer has kept in mind the fact that there has been very little published in this country on the use of the stains in combination.

METHOD OF DOUBLE STAINING

(*a*) SECTIONS.—Free-hand sections of white-pine bark can be made to answer very well if no microtome is available, but many more sections of a better quality can be obtained with an ether or carbon-dioxid freezing attachment and a small microtome. Radial longitudinal sections are best, for in them the medullary rays are exposed, and in infected bark the mycelium of the blister-rust is generally closely associated with the cells of the rays. The sections should include, where practicable, the whole width of the bark, the cambium region, and three or four annual rings of wood. Such sections are, of course, larger than can be conveniently cut with a section razor by hand, but it is perfectly possible to

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cut them with the freezing microtome if the operator is careful not to cut them too thin. The advantage in having sections of such large size lies in the fact that the observer can explore the whole of the susceptible regions for evidence of the mycelium of the parasite under conditions really favorable for its discovery.

No killing solution is used, but, so far as can be observed, no shrinkage at all occurs in the hyphæ, and but little in the host cells of fresh specimens. The blocks of bark and wood should be cut into pieces approximately ⅛ to ¼ inch on a side. The prepared blocks are then soaked for about 10 minutes in a 10 per cent aqueous solution of gum arabic containing 0.5 per cent of phenol. Blocks cut from fresh specimens can be put to soak at once. If the specimen is old and dry, the blocks should be boiled before being put into the gum arabic. The same strength gum-arabic solution is used as a freezing medium. If the material be embedded in paraffin, the usual process of sectioning and mounting can be followed. Excellent sections of young pine bark extending from cambium to epidermis can be easily cut 5 μ thick from paraffin material. However, for ordinary diagnosis or morphological study of the fungus hyphæ sections 15 to 20 μ thick, cut on the freezing microtome, are very satisfactory. Such sections should be removed from the knife with a moist camel's-hair brush and rinsed in several changes of clean water. They can be handled very conveniently in bevel-ground Syracuse watch glasses.

(*b*) STAINS.—The stains, safranin and lichtgruen, should be kept in dropper bottles of 25-to-50-c. c. capacity and used from the dropper or pipette. Similar bottles should be used for absolute alcohol, clove oil, and xylol. The writer uses the alcoholic solution of safranin almost exclusively. This method is described by Chamberlain [1] as follows:

> The alcoholic solution is made by dissolving 1 g. of the alcoholic safranin in 100 c. c. of 95 per cent or absolute alcohol, and, after the safranin is completely dissolved, adding 50 c. c. of distilled water.

This stain may be used over and over again, but it should preferably be filtered back into the drop bottle after each staining. A convenient stock solution of lichtgruen is a 1 per cent in 95 per cent alcohol. A convenient strength for use is one-half stock, made by diluting the stock with an equal volume of 95 per cent alcohol. For thin sections, however, from paraffin material, better results can be obtained by the rapid use of the stain at full strength. In addition to the regular drop bottle pipettes, there should be two or three others of good capacity and small tube opening for use in draining off the stains or excess reagents during the steps of the process.

(*c*) STAINING PROCESS.—The sections should be rinsed several times and then drained of all excess water and a generous quantity of safranin added. This stain should remain for from two to four hours, after

[1] CHAMBERLAIN, C. J. METHODS IN PLANT HISTOLOGY. ed. 3, p. 51. Chicago, 1915.

which time it may be drawn off and kept to be filtered back into the drop bottle. By tilting the watch glass the sections can be grouped easily at one side of the watch glass. Then, after the safranin has been drawn off, any excess red in the dish may be wiped out with a cloth. Care should be taken that the sections do not dry out. The lichtgruen should be added as soon as the excess safranin is removed. A muddy mixture of red and green results. The lichtgruen replaces the safranin quite rapidly, so that a minute is usually the maximum time necessary to produce the proper green tint. The writer shakes the watch glass as soon as the lightgruen is added, then draws off the red-green mixture, and adds a few drops of fresh lichtgruen. The green stain should not be allowed to act long enough to wash out too much of the safranin. The time required depends on (1) the thickness of the sections, (2) the time the sections were stained in safranin, and (3) the strength of the lichtgruen solution. The used lichtgruen should be thrown into a waste jar.

(*d*) CLEARING AND MOUNTING.—After the removal of the green stain, absolute alcohol should be added immediately and the watch glass rocked or shaken. This absolute alcohol should be drawn off and saved in a waste jar, and the sections treated with fresh absolute alcohol for a minute or two, the total time depending on the number of sections handled at one time. This absolute alcohol should then be replaced by clove oil. While in clove oil the sections may be conveniently lifted with a small brush and arranged on the slide without danger of drying up, and for this reason clove oil is used between absolute alcohol and the more volatile xylol. After the sections are arranged, they should be rinsed with drops of xylol until the clove oil has been removed. A drop of balsam and a warm cover complete the mount. It is advisable to set the slide away for a day in the paraffin bath with a heavy lead weight on the cover to flatten out the sections.

(*e*) COLOR RESULTS.—The color results in the finished slide vary slightly, depending on the amount of safranin which was removed when the green stain was acting. A good diagnostic slide for *Cronartium ribicola* should have red host nucleoli and reddish nuclei, red wood cells, dead cells, and cork cells. Occasionally resin cells stain a deep red. The host cytoplasm and all cellulose walls should be green. The hyphæ of the parasite stain green, but they stand out well when they are colored a greenish pink or greenish red—that is, when the red is not removed too far. The nuclei of the parasite should be red. Although the stains employed do not result in the differential staining of the host and parasite, there is no difficulty in distinguishing them.

(*f*) ADVANTAGES OF THE METHOD.—The chief advantages of the method are its simplicity and rapidity. Permanent record slides from a suspected specimen can be completed easily in about two and one-half hours from the time the specimens reach the laboratory. Correctly

prepared slides are sufficiently transparent for a thorough exploration with oil-immersion lenses. The practicability of the method has been demonstrated by constant use during the past year in the diagnosis of suspected cases of blister-rust and in the study of the anatomical features of parasite and host.

MYCELIUM OF THE PARASITE

The characters which distinguish the hyphæ of *Cronartium ribicola* from other hyphæ occasionally found in white-pine bark are their comparatively large size, fairly uniform diameter, and possession of haustoria. They are larger than any other parasitic hyphæ which the writer has observed in pine bark. While there is, of course, some difference in the width of individual hyphæ one rarely finds them deviating widely from the average width of 3 to 3.5 μ. The haustoria are particularly striking. They invade practically every cell in the infected region in young bark. In older bark they are most prominent in the cells of the medullary rays; and for this reason, as mentioned above, radial sections are particularly satisfactory for study. At the point where the haustoria pass through the cell wall, they are constricted. Once inside the cell their width increases until they average a little more than 4 μ in diameter (Pl. 31, B). Prominent bulges, which occur quite frequently, give them a characteristically irregular outline. When young and short they are straight; as they mature they usually develop pronounced bends, which may be as great as right angles. The host nuclei appear to attract the haustoria. Frequently their tips press dents in the sides of the host nuclei (fig. 1, a), recalling the occurrence of this well-known phenomenon in many other rusts; but, so far as the writer has been able to observe, the nucleus does not appear to suffer greatly from this close association with the parasite. In fact, the cells in which the haustoria are present seem to be quite as healthy as the cells which have remained free from attack. Usually the haustoria are unbranched; but in some instances, especially in the cells of the medullary rays, they branch (fig. 1, b). In no case have more than three tips been observed. All the cells of the mycelium, including the haustoria, are uninucleate until cell fusion occurs at the base of the æcium.

The Peronosporales and the Erysiphaceae are the only other groups of parasitic fungi beside the Uredinales which possess prominent haustoria. No members of the first two groups are known to parasitize the bark of *Pinus strobus*. Moreover, the second group is composed of leaf parasites whose mycelium is almost entirely exterior. Even if by any chance material containing mycelium from either of these two groups should be introduced into the mount along with the bark sections, there would be no danger of confusion. The fact that the members of the first group have cœnocytic hyphæ and that the members of the second group possess

haustoria which are decidedly different in appearance [1] from those of the blister-rust, rules out any possibility of confusing the mycelium of members of either of the two groups with the mycelium which has been described.

METHOD OF PARASITISM

The manner in which the parasite attacks the host is also important as a distinguishing character. The hyphæ are intercellular in position. As stated above, practically every cell of the bark may be pierced by one or more haustoria. Parenchyma cells of the cortex and in the neighborhood of the resin ducts, sieve cells, medullary-ray cells, and even resin cells are all subject to attack. Only the cork and schlerenchyma

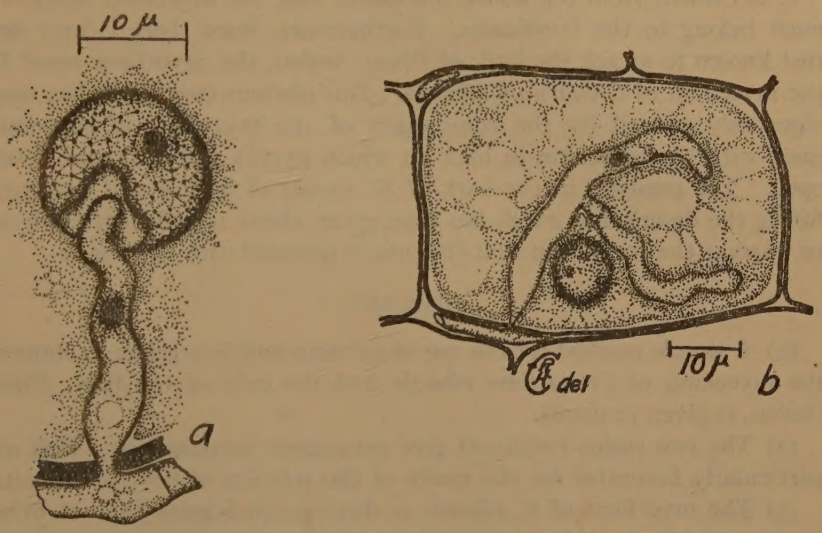


FIG. 1.—*Cronartium ribicola: a*, A moderately straight haustorium which has dented the side of the host nucleus. Note the manner in which the haustorium is constricted in passing through the cell wall. *b*, A branched haustorium in a young medullary-ray cell. The figures were drawn with the aid of a camera lucida.

cells of old bark and, of course, dead bark are apparently immune. The hyphæ may follow the medullary rays past the cambium and into the xylem for at least three annual rings, and in these medullary-ray cells the haustoria are quite as prominent as in the ray cells of the phloem. Haustoria do not enter the wood cells. The parenchyma cells surrounding the resin ducts in the last two annual rings of xylem are also heavily attacked. In tangential or radial sections one sees that the hyphæ are frequently massed in strands (Pl. 31, A). These strands are often formed in the region of the thin-walled sieve tubes, but they are also scattered through the parenchyma. Generally the medullary-ray cells are forced apart by single layers of hyphæ. Probably the swelling of the bark is

[1] SMITH, Grant. THE HAUSTORIA OF THE ERYSIPHEÆ. *In* Bot. Gaz., v. 29, no. 3, p. 153–184, pl. 11–12. 1900.

due almost entirely to the spreading action of the hyphæ as they force themselves between the cells. This method of attack differs from that of other parasitic fungi observed on white-pine bark. In attacks made by species of Phoma and Fusicoccum the cells are killed, the hyphæ run through the cells, and the bark tends to dry up, turn brown, and crack; whereas in the case of the blister-rust attack the cells remain alive for a long time, the hyphæ run between the cells, the bark swells, turns a yellowish green color, and does not crack until the æcia are produced.

VALUE OF MYCELIAL CHARACTERS, HAUSTORIA, AND METHOD OF PARASITISM IN DIAGNOSIS

It is evident from the above discussion that the mycelium described must belong to the Uredinales. Furthermore, since there is only one rust known to attack the bark of *Pinus strobus*, the mycelium must be the mycelium of *Cronartium ribicola*. This obvious conclusion has been vigorously proved by the comparison of the mycelium in suspected cases with the mycelium in bark on which pycnia and æcia had developed. The presence in the bark of *P. strobus* of hyphæ and haustoria fitting the description which has been given above is sufficient evidence to warrant the conclusion that the pine is infected with *C. ribicola*.

SUMMARY

(1) A simple method for the use of safranin and lichtgruen in staining the mycelium of *Cronartium ribicola* and the cells of the host, *Pinus strobus*, is given in detail.

(2) The two stains employed give permanent preparations which are particularly favorable for the study of the relation of host to parasite.

(3) The mycelium of *C. ribicola* is distinguished from that of other fungi parasitic on white-pine bark by the size of the hyphæ and the possession of haustoria.

(4) The manner in which the parasite attacks the host cells is described in detail. This method of attack is characteristic of the blister-rust in white-pine bark.

(5) The presence in the bark of *P. strobus* of mycelium such as has been described is sufficient evidence to warrant the conclusion that the pine is infected with *C. ribicola*.

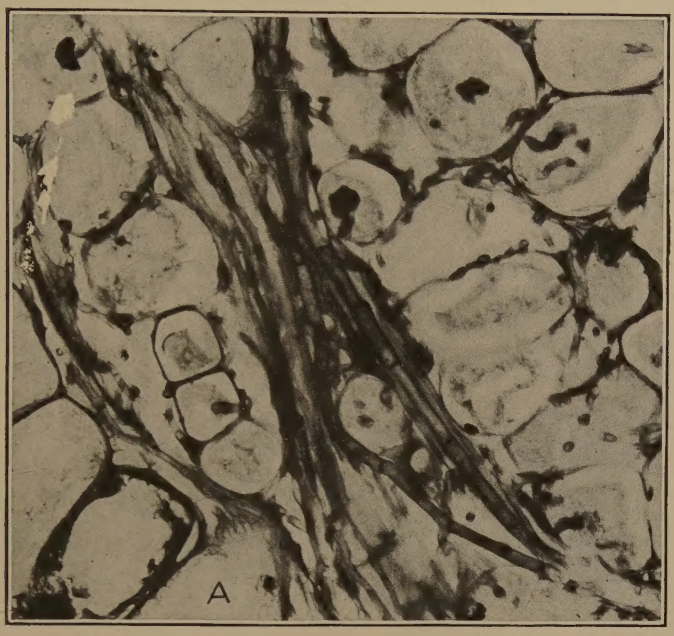
PLATE 31

Cronartium ribicola:

A.—A tangential section showing strands of hyphæ among the white-pine host cells. Note the haustoria. In the upper left-hand corner a haustorium can be seen denting the host nucleus. × 325.

B.—A haustorium showing the characteristic bend and irregular outline. The section just missed the connection of the haustorium with the hypha outside the cell. The host nucleus was torn by the razor. × 1,250.

These figures illustrate excellently the relation of the parasite to the host in the phloem parenchyma. Photomicrographs by the writer.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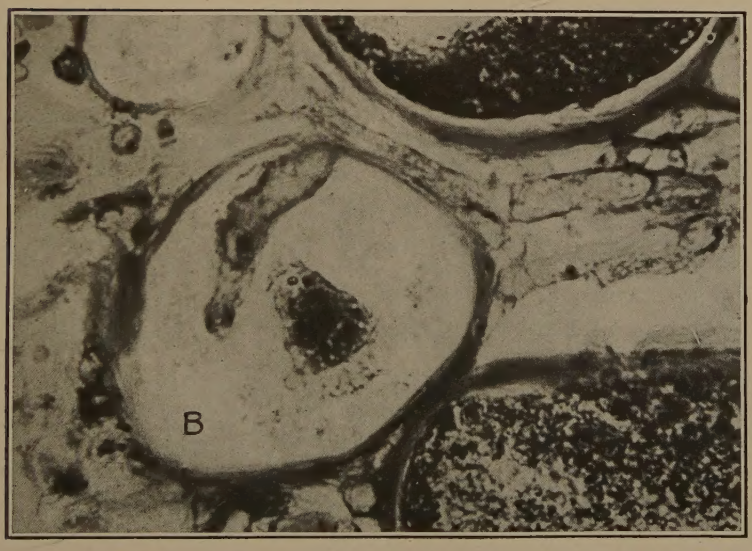
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